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(54) Title: METHOD TO OBTAIN MALE-STERILE PLANTS

(57) Abstract

A method to obtain male-sterile plants by transforming the nuclear genome of plant cells with a foreign DNA comprising a male-sterility gene comprising: a male-sterility DNA encoding a sterility RNA, protein or polypeptide, preferably barnase, which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell; and, a sterility promoter capable of directing expression of the male-sterility DNA selectively in specific stamen cells, especially in anther cells, particularly in tapetum cells, of said plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter; and regenerating plants transformed with said foreign DNA from said transformed cells, which method is characterized by including in said foreign DNA a coregulating gene comprising a coregulating DNA encoding a coregulating RNA, protein or polypeptide, preferably barstar, which is capable, when produced in plant cells wherein said sterility RNA, protein or polypeptide is produced, of sufficiently preventing the activity of said sterility RNA, protein or polypeptide, said coregulating DNA preferably being under the control of a promoter selected from the group consisting of: a promoter capable of directing expression of said coregulating DNA in non-stamen cells, preferably at least in the majority of non-stamen cells, while directing low-level expression, preferably not directing expression, in said specific stamen cells; a promoter consisting of a minimal promoter element, preferably of a promoter normally expressed in plant cells, particularly whereby said coregulating DNA is capable of being placed under control of enhancer elements in the nuclear genome of said plant after integration of said foreign DNA in said plant genome, whereby said coregulating DNA is in a different plant transcriptional unit as said sterility DNA, and provided that, when said coregulating DNA is not under control of a promoter capable of directing expression in plant cells, said coregulating gene is located in said foreign DNA in such a way that after insertion in the plant genome, the coregulating DNA is capable of being placed under the control of plant promoter sequences present in the DNA surrounding said foreign DNA in said plant genome.

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METHOD TO OBTAIN MALE STERILE PLANTS

The present invention relates to an improved method to obtain male-sterile plants using foreign male-sterility genes that comprise plant promoters that direct expression of a male-sterility DNA in stamen cells, and to plants obtained by the method.

Background to the Invention

In many, if not most plant species, the development of hybrid cultivars is highly desired because of their generally increased productivity due to heterosis: the superiority of performance of hybrid individuals compared with their parents (see e.g. Fehr, 1987, Principles of cultivar development, Volume 1 : Theory and Technique, MacMillan Publishing Company, New York; Allard, 1960, Principles of Plant Breeding, John Wiley and Sons, Inc.).

The development of hybrid cultivars of various plant species depends upon the capability to achieve almost complete cross-pollination between parents. This is most simply achieved by rendering one of the parent lines male sterile (i.e. bringing them in a condition so that pollen is absent or nonfunctional) either manually, by removing the anthers, or genetically by using, in the one parent, cytoplasmic or nuclear genes that prevent anther and/or pollen development (for a review of the genetics of male sterility in plants see Kaul, 1988, 'Male Sterility in Higher Plants', Springer Verlag).

For hybrid plants where the seed is the harvested product (e.g. corn, oilseed rape) it is in most cases also necessary to ensure that fertility of the hybrid plants is fully restored. In systems in which the male sterility is under genetic control this requires the existence and use of genes that can restore male fertility. The development of hybrid cultivars is mainly dependent on the availability of suitable and effective sterility and restorer genes.

Endogenous nuclear loci are known for most plant species that may contain genotypes which effect male sterility, and generally, such loci need to be homozygous for particular recessive alleles in order to result in a male-sterile phenotype. The presence of a dominant 'male fertile' allele at such loci results in male fertility.

Recently it has been shown that male sterility can be induced in a plant by providing the genome of the plant with a chimeric male-sterility gene comprising a DNA sequence (or male-sterility DNA) coding, for example, for a cytotoxic product (such as an RNase) and under the control of a promoter which is predominantly active in selected tissue of the male reproductive organs. In this regard stamen-specific promoters, such as the promoter of the TA29 gene of Nicotiana tabacum, have been shown to be particularly useful for this purpose (Mariani et al., 1990, Nature 347:737, European patent publication ("EP") 0,344,029). By providing the nuclear genome of the plant with such a male-sterility gene, an artificial male-sterility locus is created containing the artificial male-sterility genotype that results in a male-sterile plant. Various stamen-specific promoters have been described (see e.g. WO 92/13956, WO 92/13957).

In addition it has been shown that male fertility can be restored to the plant with a chimeric fertility-restorer gene comprising another DNA sequence (or fertility-restorer DNA) that codes, for example, for a protein that inhibits the activity of the cytotoxic product or otherwise prevents the cytotoxic product to be active in the plant cells (EP 0,412,911). For example the barnase gene of Bacillus amyloliquefaciens codes for an RNase, the barnase, which can be inhibited by a protein, the barstar, that is encoded by the barstar gene of B. amyloliquefaciens. The barnase gene can be used for the construction of a sterility gene while the barstar gene can be used for the construction of a fertility-restorer gene. Experiments in different plant species, e.g. oilseed rape, have shown that a chimeric barstar gene can fully restore the male fertility of male sterile lines in which the male sterility was due to the presence of a chimeric barnase gene (EP 0,412,911, Mariani et al., 1991, Proceedings of the CCIRC Rapeseed Congress, July 9-11, 1991, Saskatoon, Saskatchewan, Canada; Mariani et al., 1992, Nature 357:384). By coupling a marker gene, such as a

dominant herbicide resistance gene (for example the bar gene coding for phosphinothricin acetyl transferase (PAT) that converts the herbicidal phosphinothricin to a non-toxic compound [De Block et al., 1987, EMBO J. 6:2513]), to the chimeric male-sterility and/or fertility-restorer gene, breeding systems can be implemented e.g. to select for uniform populations of male sterile plants (EP 0,344,029; EP 0,412,911).

Barnase is an extracellular ribonuclease produced by Bacillus amyloliquefaciens. Barstar is an inhibitor of barnase that is produced intracellularly by the same bacterium to protect it from the toxic effects of the intracellular barnase activity (Hartley, 1989, TIBS, 14:450-454). Initial attempts to clone the barnase gene in E.coli and B.subtilis under control of its own or another bacterial promoter were unsuccesful as the produced barnase proved to be toxic to the host cells. When the barnase gene was reconstructed from previously cloned parts on the same plasmid as the barstar gene, the lethal effects of barnase expression were suppressed (Hartley, 1988, J.Mol.biol. 202:913-915).

Whenever barnase is cloned in a bacterial host cell, such as E.coli, it may be useful to have the barstar gene, under control of its native or another bacterial promoter, present in the host cell to prevent possible harmful effects of undesired barnase expression. Paul et al, 1992, Plant Mol. Biol. 19:611-622 for instance, constructed a chimeric barnase gene under control of a tapetum specific promoter of the A9 gene of Arabidopsis. Plasmids pWP127 and pWP128 contain a DNA fragment encoding barstar and the mature barnase cloned between the 1437 bp A9 promoter fragment and a CaMV polyadenylation sequence. The promoter and coding sequence of barstar were included on these plasmids since mature barnase could not be cloned in its absence in E.coli.

As indicated above barnase DNA has been used to induce male-sterility in plants. However, other uses of barnase have also been described. WO 92/21757 describes inter alia a plant transformed with a nematode-induced chimaeric gene comprising the following operably linked DNA sequences:

- a nematode-induced promoter that is suitable to direct transcription of a foreign DNA substantially selectively in specific root cells, preferably in the cells of fixed-feeding sites of the plant; and,
- a first foreign DNA that encodes barnase;

and which also contains a restorer chimaeric gene, preferably in the same genetic locus as the nematode-induced chimaeric gene, comprising the following operably linked DNA sequences:

- a second promoter, such as a nematode-repressed promoter, which can direct transcription of a second foreign DNA in cells of the plant where the first foreign DNA is expressed, preferably substantially selectively in cells other than the specific root cells, preferably in cells other than the fixed feeding site cells, of the plant, and,
- a second foreign DNA that encodes barstar.

WO 93/19188 describes inter alia a plant transformed with a fungus-responsive chimaeric gene comprising the following operably linked DNA sequences:

- a fungus-responsive promoter that is suitable to direct transcription of a foreign DNA substantially selectively in cells of a plant surrounding, preferably immediately surrounding, a site of infection of the plant by a fungus; and,
- a first foreign DNA that encodes barnase;

and which also contains a restorer chimaeric gene, preferably in the same genetic locus as the fungus-responsive chimaeric gene, comprising the following operably linked DNA sequences:

- a second promoter, such as a constitutive promoter (e.g. 35S), which can direct transcription of a second foreign DNA in cells of the plant other than those surrounding, preferably in at least cells of the plant other than those immediately surrounding, said fungus infection site; and,
- a second foreign DNA that encodes barstar.

A foreign DNA, when introduced in the plant genome appears to integrate randomly in the plant genome. Examination of independently transformed plants has shown a high degree of variability (up to 100-fold) in the expression level of the introduced gene. Several studies have shown no correlation between this "between-transformant variability" and the copy number of the introduced DNA at a given locus.

It has been suggested that some of the variability in expression of introduced genes in transgenic plants is a consequence of "position effects" caused by influences of adjacent plant genomic DNA. Other factors that could contribute to the variability in expression are physiological variability of the plant material, differences in the number of independent T-DNA loci in different transformants or the inhibitory effects of certain T-DNA structures on gene expression. Between-transformant variability in expression has been observed for the majority of introduced genes in transgenic plants. The variability in expression of many introduced genes in independent transgenic plants necessitates large numbers of transgenic plants to be assayed to accurately quantitate the expression of the gene. It would be of great importance if the amount of between-transformant variability could be reduced (Dean et al, 1988, NAR 16:9267-9283).

Summary of the Invention

The invention concerns a plant having in the nuclear genome of its cells foreign DNA comprising :

- a male-sterility gene comprising:
  - a male-sterility DNA encoding a sterility RNA, protein or polypeptide which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell, and,
  - a sterility promoter directing expression of the male-sterility DNA selectively in specific stamen cells, especially in anther cells, particularly in tapetum cells, of the plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter; and
- a coregulating gene comprising:
  - a coregulating DNA encoding a coregulating RNA, protein or polypeptide which is capable, when produced in plant cells wherein the sterility RNA, protein or polypeptide is produced, of sufficiently preventing the activity of the sterility RNA, protein or polypeptide, and preferably

- a promoter directing expression of said coregulating DNA in non-stamen cells, preferably at least in the majority of non-stamen cells, while directing low-level expression, preferably not directing expression, in said specific stamen cells, or
- a promoter consisting of a minimal promoter element, preferably of a promoter normally expressed in plant cells, particularly whereby said coregulating DNA is under control of enhancer elements in the nuclear genome of said plant,

whereby the coregulating DNA is in a transcriptional unit which is different from the transcriptional unit of the sterility DNA.

This invention also provides a method to obtain male-sterile plants which comprises :

- transforming the nuclear genome of plant cells with a foreign DNA comprising a male-sterility gene comprising:
  - a male-sterility DNA encoding a sterility RNA, protein or polypeptide, preferably barnase or a variant thereof, which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell, and,
  - a sterility promoter capable of directing expression of the male-sterility DNA selectively in specific stamen cells, especially in anther cells, particularly in tapetum cells, of said plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter, and
- regenerating plants transformed with said foreign DNA from said transformed cells,

which method is characterized by including in said foreign DNA a coregulating gene comprising a coregulating DNA encoding a coregulating RNA, protein or polypeptide, preferably barstar, which is capable, when produced in plant cells wherein said sterility RNA, protein or polypeptide is produced, of sufficiently preventing the activity of said sterility RNA, protein or polypeptide, said coregulating DNA preferably being under the control of a promoter including :

- a promoter capable of directing expression of said coregulating DNA in non-stamen cells, preferably at least in the majority of non-stamen cells, while directing low-level expression, preferably not directing expression, in said specific stamen cells,

- a promoter consisting of a minimal promoter element, preferably of a promoter normally expressed in plant cells, particularly whereby said coregulating DNA is capable of being placed under control of enhancer elements in the nuclear genome of said plant after integration of said foreign DNA in said plant genome,

whereby said coregulating DNA is in a plant transcriptional unit which is different from the plant transcriptional unit of said sterility DNA, and provided that, when said coregulating DNA is not under control of a promoter capable of directing expression in plant cells, said coregulating gene is located in said foreign DNA in such a way that after insertion in the plant genome, the coregulating DNA is capable of being placed under the control of plant promoter sequences present in the DNA surrounding said foreign DNA in said plant genome.

The present invention further provides plants that contain in their nuclear genome said male-sterility gene and said coregulating gene, preferably in the same genetic locus.

Description of the invention

A male-sterile plant is a plant of a given plant species which is male-sterile due to expression of a male-sterility genotype such as a foreign male-sterility genotype containing a male-sterility gene. A restorer plant is a plant of the same plant species that contains within its genome at least one fertility-restorer gene that is able to restore the male fertility to a line of male-sterile plants containing a male-sterility genotype i.e. in those offspring obtained from a cross between a male-sterile plant and a restorer plant and containing both a male-sterility genotype and a fertility-restorer gene. A restored plant is a plant of the same species that is male-fertile and that contains within its genome a male-sterility genotype and a fertility-restorer gene.

A line is the progeny of a given individual plant.

A gene as used herein is generally understood to comprise at least one DNA region coding for an RNA, which may or may not be capable of being translated into a protein or polypeptide, which is operably linked to regulatory sequences that

control the transcription of the DNA region. Such regulatory sequences include promoter regions, enhancer sequences and 3' regulatory sequences. A structural gene is a gene whose product is e.g. an enzyme, a structural protein, tRNA or rRNA. A regulatory gene is a gene which encodes a protein which regulates the expression (e.g. the transcription) of one or more structural or other regulatory genes.

For the purpose of this invention the expression of a gene (or of a DNA of the gene which encodes the RNA), such as a chimeric gene, means that the DNA region of the gene coding for the RNA is transcribed, under control of the promoter and other regulatory sequences of the gene, into a RNA which is biologically active i.e. which is either capable of interacting with another RNA, or which is capable of being translated into a biologically active polypeptide or protein.

The expression of most eucaryotic genes, including foreign (e.g. chimeric) genes, is regulated by combination of a minimal promoter element and one or more enhancer elements which bind to regulatory proteins. When a promoter directs expression of any DNA it is active. Depending on the amount of RNA produced by a promoter under a given set of conditions one can speak about low or high level of expression (or less or high activity of the promoter). With regard to the present invention a "high" level of expression of the male-sterility gene is interpreted as the level of expression in specific stamen cells whereby the production of fertile male gametes is prevented.

A minimal promoter element as used herein means a DNA that has the capacity to bind RNA polymerase and to inititate transcription. For any given gene the minimal promoter extends about 30-40, maximally 100, basepairs upstream from the transcription initiation site and generally includes the TATA box. An enhancer element is a regulatory element that is generally further upstream from the minimal promoter and that activates (or inhibits) transcription from the minimal promoter linked to it, with synthesis beginning at the normal start site. An enhancer is capable of binding transcription factors and can usually operate in both orientations and can function even when moved more than 1000 basepairs from the promoter and from either an upstream or a downstream position.

A promoter as used herein comprises a minimal promoter associated with one or more enhancer elements. For practical purposes a promoter and minimal promoter,

as used herein, may also comprise part of the DNA that is transcribed (e.g. the untranslated leader of a mRNA).

A transcriptional unit means a DNA segment that is transcribed into a continuous RNA from a promoter. For the purposes of this invention a transcriptional unit comprises the promoter.

A promoter which directs expression selectively in specific cells or tissues of a plant (e.g. stamen cells such as tapetum cells) is a promoter in which the enhancer elements operate to limit the transcription to specific cells or tissues in the plant and/or to specific stages of development of these specific cells or tissues, i.e. to enhance transcription in the specific cells or tissues at particular developmental stages and to inhibit transcription in all other cells or tissues or at other developmental stages. For all practical purposes such selective promoters are specific in activity and effect. Usually such selective promoters are identified by differential screening of mRNA libraries from different tissues (Sambrook et al., 1989, "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, and Ausubel et al, 1994, "Current Protocols in Molecular Biology", John Wiley & Sons). Although it is generally impossible to screen all tissues and all cells of a plant, promoters obtained in this way have been found to be useful to direct expression of heterologous DNA selectively in the same tissues in transgenic plants of the same and/or different plant species.

As used herein stamen cells will mean cells of at least one part of the male reproductive organ in a flower, in various stages of development, such as the filament, the anther, the tapetum, the anther cell wall, the pollen etc. A stamen-specific promoter is a promoter that is capable of directing expression (e.g. of barnase DNA) selectively in stamen cells (preferably including at least tapetum cells) at one or more stages in the development of the stamen to prevent the production of fertile pollen. It should be noted that a male-sterility gene comprising a pollen-specific promoter, i.e. a promoter that directs expression exclusively in microspores and/or pollen (i.e. after meiosis), when operably linked to a barnase DNA can only induce male-sterility in a plant when it is present in a homozygous form in the nuclear genome of that plant.

Non-stamen cells as used herein means all cells of a plant except the stamen cells (particularly the tapetum cells), especially those stamen cells in which the sterility promoter can direct expression of the barnase DNA.

The phenotype is the external appearance of the expression (or lack of expression) of a genotype i.e. of a gene or set of genes (e.g. male-sterility, presence of protein or RNA in specific plant tissues etc.).

As used herein, a genetic locus is a DNA (e.g. one or more genes) as defined with respect to its position in the nuclear genome, i.e. in a particular chromosome, of a plant. Two loci can be on different chromosomes and will segregate independently. Two loci can be located on the same chromosome and are then generally considered as being linked (unless sufficient recombination can occur between them).

An endogenous locus is a locus which is naturally present in a plant species. A foreign locus is a locus which is formed in the plant because of the introduction, e.g. by means of genetic transformation, of a foreign DNA. If a foreign DNA, which comprises two or more genes, is introduced in the plant genome this will generally be regarded as creating, in the plant genome, one foreign locus which comprises the two or more genes (although it can also be said that two or more closely linked loci are created).

In diploid plants, as in any other diploid organisms, two copies of a gene are present at any autosomal locus. Any gene can be present in the nuclear genome in several variant states designated as alleles. If two identical alleles are present at a locus that locus is designated as being homozygous, if different alleles are present, the locus is designated as being heterozygous. The allelic composition of a locus, or a set of loci, is the genotype. Any allele at a locus is generally represented by a separate symbol (e.g. M and m, S and -, - representing the absence of the gene). A foreign locus is generally characterized by the presence and/or absence of a foreign DNA. A dominant allele is generally represented by a capital letter and is usually associated with the presence of a biologically active gene product (e.g. a protein) and an observable phenotypic effect.

A plant can be genetically characterized by identification of the allelic state of at least one genetic locus.

The genotype of any given locus can be designated by the symbols for the two alleles that are present at the locus (e.g. M/m or m/m or S/-). The genotype of two unlinked loci can be represented as a sequence of the genotype of each locus (e.g. S/S,R/-)

Foreign male-sterility loci are those in which the allele responsible for male sterility is a foreign DNA sequence S which comprises the male-sterility gene which when expressed in cells of the plant renders the plant male-sterile without otherwise substantially affecting the growth and development of the plant.

The male-sterility locus preferably also comprises in the same genetic locus at least one marker gene T which comprises at least:

t1) a marker DNA encoding a marker RNA, protein or polypeptide which, when present at least in a specific tissue or specific cells of the plant, renders the plant easily separable from other plants which do not contain the marker RNA, protein or polypeptide encoded by the marker DNA at least in the specific tissue or specific cells, and,

t2) a marker promoter capable of directing expression of the marker DNA at least in the specific tissue or specific cells: the marker DNA being in the same transcriptional unit as, and under the control of, the marker promoter.

Such male-sterility gene is always a dominant allele at such a foreign male-sterility locus. The recessive allele corresponds to the absence of the male-sterility gene in the nuclear genome of the plant.

Male-sterility DNAs and sterility promoters that can be used in the male-sterility genes of this invention have been described before (EP 0,344,029 and EP 0,412,911). For the purpose of this invention the expression of the male-sterility gene in a plant cell should be able to be inhibited or repressed for instance by means of expression of a suitable fertility-restorer gene in the same plant cell. In this regard a particular useful male-sterility DNA codes for barnase (Hartley, J.Mol. Biol. 1988 202:913). The sterility promoter can be any promoter but it should at least be active in stamen cells, particularly tapetum cells. Particularly useful sterility promoters are promoters that are selectively active in stamen cells, such as the tapetum-specific promoters of the TA29 gene of Nicotiana tabacum (EP 0,344,029) which can be used

in tobacco, oilseed rape and other Brassica species, cichory, corn, rice, wheat and other plant species; the PT72, the PT42 and PE1 promoters from rice which can be used in rice, corn, wheat, and other plant species (WO 92/13956) ; the PCA55 promoter from corn which can be used in corn, rice, wheat and other plant species (WO 92/13957); and the A9 promoter of a tapetum-specific gene of Arabidopsis thaliana (Paul et al., 1992, Plant Mol. Biol. 19:611-922).

It has been found that stamen-specific promoters, such as PTA29, operably linked to a suitable sterility DNA, such as the barnase DNA, can be used in a variety of plant species to induce male-sterility. Indeed, by transformation of plants with such male-sterility genes, male-sterile lines with high agronomic value have been obtained in many plant species. Apparently, the stamen-specific promoters, for all practical purposes, substantially retain their spatial and temporal specificity. However, not all individual transformed plants can be developed into lines with good agronomical performance. Indeed some plants show undesired phenotypic effects which can be due to somaclonal variation and/or 'position effects'. It is believed that at least part of this variation is due to the regulating effects of native (i.e. endogenous) enhancer elements in the plant genome that surround the integrated male-sterility gene in the transgenic plants. Such enhancer sequences, and consequently their effects on the expression of the male-sterility gene, differ depending on the place of integration of the male-sterility gene. This can result, in some transformants, in low-level (often even undetectable) expression of the sterility DNA (e.g. barnase DNA) in tissues other than the stamen cells, e.g. in cells during tissue culture or in somatic cells of the plants or seeds.

In this regard, this invention is based on the observation that, under some circumstances, a chimeric gene such as the barstar gene, introduced together with a male-sterility gene such as a gene comprising barnase DNA can decrease the between-transformant variability in expression of the male-sterility gene, and of its resulting phenotype, and can increase the frequency of transformants having good agronomical performance. For the purposes of this invention it is therefore preferred that the sterility DNA is the barnase DNA while the coregulating DNA is the barstar DNA.

For the purposes of this inv ntion barnase DNA means a DNA coding for the ribonuclease of Bacillus amyloliquefaciens with the amino acid sequence as described by Hartley, 1988, J.Mol.Biol. 202:913-915 (barnase s.s.) or any variants thereof which have ribonuclease activity and are capable of being inactivated by barstar. In this regard one of such variants of barnase s.s. has been found to be encoded by the DNA of Bacillus intermedius which encodes a ribonuclease (binase) which has 84% identity at the amino acid level with barnase s.s. (Schulga et al, 1992, NAR 20:2375; see also Guillet et all, 1993, Structure 1:165-177). Preferably, the barnase variants retain at least 10% particularly at least 50% of the activity of barnase s.s. as measured under standard conditions (Fitzgerald and Hartley. 1993, Anal. Biochem. 214:544-547; Hartley et al, 1993, Biochemistry 32:5978-5984).

For the purposes of this invention barstar DNA means a DNA coding for an inhibitor of the barnase ribonuclease of Bacillus amyloliquefaciens as described by Hartley, 1988, J.Mol.Biol. 202:913-915 (barstar s.s.) or any variants thereof which are capable of inhibiting barnase s.s. In this regard one of such variants has been found to be encoded by the DNA Bacillus intermedius which encodes binstar (Guillet et al, 1993, Structure 1:165-177). Preferably the barstar variants are capable of inhibiting at least 90% of barnase activity, particularly at least 50% of barnase activity, in an equimolar mixture of the barstar variant and barnase in standard condition (Hartley et al, 1993, Biochemistry 32:5978-5984).

However, any DNA coding for a ribonuclease can be used as sterility DNA in this invention provided a DNA coding for protein inhibitor of that ribonuclease can be obtained. Examples of such RNAses and corresponding inhibitors are for instance listed in Guillet et al, 1993, Structure 1:165-177. Another example of such a ribonuclease is the RNAse Sa or sarnase of Streptomyces aureofaciens (Shlyapnikov et al, 1986, FEBS Letters 209:335-339; Homerova et al, 1992, Gene 119:147-148). An inhibitor of RNAse Sa is known (Mucha et al, 1983, Biologia 38:1177-1184).

Of course, any sterility DNA coding for a RNA, protein or polypeptide and its corresponding coregulating DNA coding for a coregulating RNA, protein or polypeptide which, when expressed in the same plant cell as the sterility DNA is

capable of preventing expression of the sterility DNA or the activity of the sterility RNA, protein or polypeptide can be used. In this regard DNAs that are described as fertility restorer DNAs in EP 0,412,911 can be used as coregulating DNAs of this invention in combination with their corresponding sterility DNAs which are also described in EP 0,412,911.

The promoter in the coregulating gene (the "coregulating promoter") of this invention is preferably capable of driving expression of the coregulating DNA (e.g. the barstar DNA) in a variety of cells and tissues, preferably all cells and tissues, of the plant to counteract the undesired effects of possible low level expression of the male-sterility gene (e.g. comprising the barnase DNA). In this regard, the promoter can also drive expression in those stamen cells in which the sterility promoter drives expression of barnase (as an example of a sterility DNA) and which are killed by the biological activity of the barnase which prevents the production of fertile male gametes. Of course in such stamen cells the activity of the sterility promoter and the coregulating promoter should be such that for instance the amount of produced barnase in such stamen cells is higher than that of the produced barstar at least during a period in stamen development. In this regard it is preferred that the coregulating promoter is not active in the same stamen cells as the sterility promoter. However, outside the stamen cells (e.g. the tapetum) in which the sterility promoter drives expression of the barnase DNA, the coregulating promoter may be active at any level. If the coregulating promoter is active in the same stamen cells as the sterility promoter (but so that sufficient barnase is still produced in the stamen cells to render the plant male-sterile) this can have the added advantage that the restoration of male fertility in the progeny of these male-sterile plants after crossing with restorer plants containing a fertility-restorer gene (e.g. comprising the barstar DNA under control of a stamen-specific promoter), is generally easier due to the fact that the amount of barnase in the stamen cells is already reduced due to expression of the coregulating gene.

Preferably the coregulating promoter is a promoter operable in plant cells and such many promoters can be used in this invention (see e.g. Fig. 1, A). In a preferred embodiment the 35S promoter ("P35S") of the Cauliflower Mosaic virus is used. This

is a family of promoters that are generally known as constitutive promoters but that appear to be relatively less active in anther cells, particularly in tapetum cells. Surprisingly it was found that the activity of the P35S is sufficiently low in tapetum cells and that it can be used together with a male-sterility gene comprising a tapetum-specific promoter. Even more surprisingly it was found that the use of the P35S as coregulating promoter was particularly effective in rice, especially when PT72 and pE1 are used as sterility promoters, and in corn, especially when PCA55 or PTA29 are used as sterility promoters.

Suitable P35S promoters can be obtained from the Cauliflower Mosaic Virus ("CaMV") isolates CM1841 (Gardner et al (1981) Nucl. Acids. Res. 9:2871) and CabbB-S (Franck et al (1980) Cell, 21:285) (the "35S2 promoter" or "P35S2"), from the CaMV isolate CabbB-JI (Hull and Howell (1978) Virology 86:482) (the "35S3 promoter" or "P35S3"). P35S3 differs from P35S2 in its sequence (the sequence of P35S3 is disclosed in European patent publication ("EP") 359617) and in its greater activity in transgenic plants (Harpster et al (1988) Mol. Gen. Genet. 212:182).

Of course other known constitutive promoters can be used as coregulating promoter. For instance the promoter of the nopaline synthase gene of Agrobacterium T-DNA ("Pnos") is known to drive low-level expression in a constitutive way in plants. It is believed that Pnos is particularly effective as coregulating promoter in dicot plants, such as Brassica species, e.g. Brassica napus.

Other suitable constitutive promoters that can be used as coregulating promoters are the TR1' and the TR2' promoters (resp. "PTR1" and "PTR2") which drive the expression of the 1' and 2' genes, respectively, of the T-DNA of Agrobacterium (Velten et al (1984) EMBO J. 3:2723), and are wound-induced promoters that are only weakly active in the uninduced state.

Suitable organ-specific, tissue-specific and/or inducible foreign promoters can also be used as coregulating promoters such as the promoters of the small subunit genes (such as the 1A gene) of 1,5-ribulose bisphosphate carboxylase of Arabidopsis thaliana (the "ssu" promoter) which are light inducible promoters (Krebbers et al (1988) Plant Mol. Biol. 11:745) active primarily in photosynthetic tissue; and the seed-specific promoters of, for example, Arabidopsis thaliana

(Krebbers et al (1988) Plant Physiol. 87:859), and the promoter of the Kunitz trypsine inhibitor gene (Jofuku and Goldberg, 1989, The Plant Cell 1:1079-1093).

In another preferred embodiment of this invention (see e.g. Fig. 1, B) the coregulating promoter comprises a minimal promoter element which can be derived from any promoter that can be expressed in plant cells including constitutive promoters (P35S, Pnos), tissue-specific promoters (PTA29, PCA55, PT72, PE1, PT42), or inducible promoters (e.g. PTR1, PTR2, Pssu). Such minimal promoter element is the sequence comprising about 30-50, maximally about 100 basepairs upstream from the transcription start site and which contains the TATA box.

Such a minimal promoter element can be used in the coregulating gene of this invention to direct low-level transciption of the barstar DNA in non-stamen cells.

In addition, the position effects in transgene expression can now be used to good effect. Indeed, the plant genomic DNA that is adjacent to the foreign DNA (or transgene) may comprise additional sequences, such as enhancer sequences, that are capable of regulating the minimal promoter to enhance transcription of the barstar DNA in a variety of plant cells. In this regard it is preferred that the coregulating gene is provided in a transforming DNA in such a way that especially upstream sequences are brought in optimal position to the minimal promoter. In this regard it is preferred that the coregulating gene is present at the extreme ends of the foreign DNA (e.g. the T-DNA).

The coregulating gene may even be lacking sequences required for being transcribed in a plant cell. For instance the coregulating gene may only comprise the coregulating DNA or it may comprise the coregulating DNA with upstream sequences that are not capable of directing expression of the coregulating DNA in plant cells. Thus the coregulating gene may lack a suitable promoter or it may comprise a bacterial promoter. (e.g. the native promoter of the barstar gene in B amyloliquefaciens or the tac promoter) (see e.g. Fig. 1, C). However, in this instance, it is preferred that the coregulating gene is present at the extreme ends of the foreign DNA used for plant transformation (e.g. the T-DNA) in such an orientation that the translation initiation codon of the coregulating DNA is closest to one of the ends of the foreign DNA. Indeed, it is believed that this orientation increases the probability

that the coregulating gene, when inserted in the plant genome, is placed under control of (i.e. has "captured") suitable promoter (e.g. minimal promoters) and/or enhancer sequences in the adjacent plant genomic DNA to enable the more or less constitutive expression of the coregulating DNA such as the barstar DNA. Because it is unlikely that the plant promoter and/or enhancer sequences will be optimally positioned with respect to the barstar DNA, it is expected that the level of any expression of the barstar DNA will be very low, as desired in many cases.

The male-sterility gene and the coregulating gene are preferably inserted in the plant genome as a single transforming DNA. Therefore both genes should preferably be present on the same vector or should be part of the same T-DNA.

However, both genes could also be present on separate DNAs which are both used for transformation. In such "cotransformation" it has been found that both DNAs are likely to be integrated in the same genetic locus of the plant genome, although there is of course a probability that both genes are integrated at different locations in the plant genome. In this respect the foreign DNA used for transformation of the nuclear genome of a plant cell need not be a single DNA molecule but can be multiple DNA molecules. For the purpose of the present invention it is however preferred that the male-sterility gene and the coregulating gene be integrated in the same locus in the plant nuclear genome.

However, if the coregulating gene is useful to counteract the low level expression of the male-sterility gene in tissue culture, its presence might not be required in the mature plants and their progeny. If the plants are transformed by cotransformation, and if the male-sterility gene and coregulating gene are integrated at different locations in the plant genome, then both genes will segregate in the progeny and the coregulating gene can hereby be removed from the transformed plant line.

The male sterile plants of this invention can be crossed with male-fertile parent plants, particularly a male-fertile restorer plant containing a suitable fertility restorer gene (see e.g. EP 0,412,911)

Marker DNAs and marker promoters that can be used in the marker gene as used in this invention are also well known (EP 0,344,029; EP 0,412,911).

Foreign DNA such as the male-sterility gene, the fertility-restorer gene, the coregulating gene, or the marker gene preferably also are provided with suitable 3' transcription regulation sequences and polyadenylation signals, downstream (i.e. 3') from their coding sequence i.e. respectively the fertility-restorer DNA, the male-sterility DNA, the coregulating DNA or the marker DNA. In this regard either foreign or endogenous transcription 3' end formation and polyadenylation signals suitable for obtaining expression of the chimeric gene can be used. For example, the foreign 3' untranslated ends of genes, such as gene 7 (Velten and Schell (1985) Nucl. Acids Res. 13:6998), the octopine synthase gene (De Greve et al., 1982, J.Mol. Appl. Genet. 1:499; Gielen et al (1983) EMBO J. 3:835; Ingelbrecht et al., 1989, The Plant Cell 1:671) and the nopaline synthase gene of the T-DNA region of Agrobacterium tumefaciens Ti-plasmid (De Picker et al., 1982, J.Mol. Appl. Genet. 1:561), or the chalcone synthase gene (Sommer and Saedler, 1986, Mol.Gen.Genet. 202:429-434), or the CaMV 19S/35S transcription unit (Mogen et al., 1990, The Plant Cell 2:1261-1272) can be used.

The fertility-restorer gene, the male-sterility gene, the coregulating gene or the marker gene in accordance with the present invention are generally foreign DNAs, preferably foreign chimeric DNA. In this regard "foreign" and "chimeric" with regard to such DNAs have the same meanings as described in EP 0,344,029 and EP 0,412,911.

The cell of a plant, particularly a plant capable of being infected with Agrobacterium such as most dicotyledonous plants (e.g. Brassica napus) and some monocotyledonous plants, can be transformed using a vector that is a disarmed Ti-plasmid containing the male-sterility gene and/or the coregulating gene (preferably both) and carried by Agrobacterium. This transformation can be carried out using the procedures described, for example, in EP 0,116,718 and EP 0,270,822. Preferred Ti-plasmid vectors contain the foreign DNA between the border sequences, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for example, in EP 0,233,247), pollen mediated transformation (as described, for example, in EP

0,270,356, PCT patent publication "WO" 85/01856, and US patent 4,684,611), plant RNA virus-mediated transformation (as described, for example, in EP 0,067,553 and US patent 4,407,956) and liposome-mediated transformation (as described, for example, in US patent 4,536,475). Cells of monocotyledonous plants such as the major cereals including corn, rice, wheat, barley, and rye, can be transformed (e.g. by electroporation) using wounded or enzyme-degraded intact tissues capable of forming compact embryogenic callus (such as immature embryos in corn), or the embryogenic callus (such as type I callus in corn) obtained thereof, as described in WO 92/09696. In case the plant to be transformed is corn, other recently developed methods can also be used such as, for example, the method described for certain lines of corn by Fromm et al., 1990, Bio/Technology 8:833; Gordon-Kamm et al., 1990, Bio/Technology 2:603 and Gould et al., 1991, Plant Physiol. 95:426. In case the plant to be transformed is rice, recently developed methods can also be used such as, for example, the method described for certain lines of rice by Shimamoto et al., 1989, Nature 338:274; Datta et al., 1990, Bio/Technology 8:736; and Hayashimoto et al., 1990, Plant Physiol. 93:857.

The transformed cell can be regenerated into a mature plant and the resulting transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the male-sterility gene, the coregulating gene (or both), in other varieties of the same related plant species. Seeds obtained from the transformed plants contain the chimeric gene(s) of this invention as a stable genomic insert. Thus the male-sterility gene, and/or the coregulating gene of this invention when introduced into a particular line of a plant species can always be introduced into any other line by backcrossing.

The present invention thus provides a method to obtain male-sterile plants whereby the frequency of obtaining, from transformation, male-sterile plants with good agronomic performance is increased. This is because the coregulating gene is expressed in non-stamen cells. In this regard the presence of the coregulating gene may counteract a number of phenomena such as:

- low-level expression of the male-sterility gene in some transformed plant cells in tissue culture, including regeneration prior to normal plant development. Indeed

such tissue culture cells have a physiology and metabolism and patterns of gene regulation which may be different from that of any differentiated cell in a plant or seed. Since the sterility promoter is generally selected on the basis of its natural activity in the plant or seed, position effects are perhaps expected to be more pronounced to activate the promoter in tissue culture cells. When direct gene transfer is used an additional phenomenon may occur. Indeed in such transformation method a large amount of DNA is delivered to any recipient cell. If gene repression should be an active process, which requires for instance DNA methylation or repressor protein binding, the repression mechanism may become temporarily overloaded, and the delivered DNA may be expressed for a short period of time. It can be seen that the coregulating gene can thus increase the general transformation efficiency.

- low-level expression of the sterility DNA (e.g. the barnase DNA) in specific non-stamen cells of the primary transformants and/or particularly the progeny plants obtained thereof. Such low level expression can be due to several factors many of which are largely unknown:
  - activation of the stamen-specific promoter by elements in the vector used for transformation,
  - position effects as outlined above. Such effects may possibly be more pronounced in plants with a small genome and little repetitive DNA, such as rice.
  - rearrangements in additional copies of the transgene. This is most likely to occur in transformation by direct gene transfer in which multiple copies of the transforming DNA are often integrated at the same genetic locus in the plant genome with subsequent rearrangements of some of the copies. During such rearrangements, a DNA containing barnase DNA could be inadvertently be placed under control of a promoter present in the transforming DNA (e.g. the P35S promoter) or in the adjacent plant genomic DNA.

Whatever the reason, the use in plant transformation of a coregulating gene of this invention combined with a corresponding male-sterility gene will generally result in a higher frequency of male-sterile transgenic plants with good agricultural performance.

It will also be appreciated that the coregulating genes of the invention will be useful in combination with a pseudo male-sterility gene which comprises a male-sterility DNA that is under control of promoters that are not entirely stamen-specific, but that also are known to direct expression in some other tissue(s) outside the stamen (e;g. seeds). In this regard the coregulating promoter should be a promoter that is active in these some other tissue(s) to a sufficient level to counter the expression of the pseudo male-sterility gene but that is does not prevent the pseudo male-sterility gene" to be expressed in stamen cells (e.g. tapetum, anther-epidermal cells). In this regard the pseudo male-sterility gene and the coregulating gene together will be equivalent to a male-sterility gene which comprises a true stamen-specific promoter.

As already indicated, the invention allows the generation of a higher number of male-sterile plants with good agronomical performance. In such plants the male-sterility gene will be genetically stable, i.e. the gene should be inherited and all plants comprising the gene should be male-sterile. Nevertheless it may not be absolutely required that all seeds that contain the gene are viable (i.e. will grow into normal mature plants). It is generally sufficient that from each male-sterile plant viable seeds that have inherited the male-sterility gene can be obtained.

Preferably the performance of the male-sterility gene (i.e. its phenotypic expression) should also be independent on genetic background so that the gene can be readily introduced in other lines through backcrossing.

It is generally also required that the male-sterility genotype is environmentally stable and that the phenotype will be independent of the various environmental conditions that can occur in the area and period in which the plants will be grown. Such environmental stability is usually demonstrated by performing field trials with the male-sterile plants in 3 or 4 different locations.

It is generally also desired that the male-sterility genotype has no significant negative effects on agronomically important characteristics and on plant development. Nevertheless this will depend not only on the performance of the male-sterile parent line, but also of the performance of the hybrid obtained from that parent line. Indeed, these negative effects can in some circumstances be compensated by significant advantages in the hybrid.

Finally, in plant species where restoration of fertility is required, either in maintenance of the male-sterile line, or in hybrid seed production, the male-sterility genotypes should be restorable by at least one fertility restorer gene.

Unless otherwise indicated all experimental procedures for manipulating recombinant DNA were carried out by the standardized procedures described in Sambrook et al., 1989, "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, and Ausubel et al, 1994, "Current Protocols in Molecular Biology", John Wiley & Sons, Vols 1 and 2.

The polymerase chain reactions ("PCR") were used to clone and/or amplify DNA fragments. PCR with overlap extension was used in order to construct chimeric genes (Horton et al, 1989, Gene 77:61-68; Ho et al, 1989, Gene 77:51-59).

All PCR reactions were performed under conventional conditions using the Vent™ polymerase (Cat. No. 254L - Biolabs New England, Beverley, MA 01915, U.S.A.) isolated from Thermococcus litoralis (Neuner et al., 1990, Arch.Microbiol. 153:205-207). Oligonucleotides were designed according to known rules as outlined for example by Kramer and Fritz (1987, Methods in Enzymology 154:350), and synthesized by the phosphoramidite method (Beaucage and Caruthers, 1981, Tetrahedron Letters 22:1859) on an Applied Biosystems 380A DNA synthesizer (Applied Biosystems B.V., Maarssen, Netherlands).

In the description and in the following examples, reference is made to the following figure and sequence listing:

FIGURES

Figure 1 : Schematic presentation of three examples of foreign DNA of this invention:

Pster : sterility promoter

barnase : region coding for barnase (as example of a sterility DNA)

3'end : 3' untranslated region of a gene (e.g. 3'nos, 3'g7)

Pcoreg : coregulating promoter (e.g. P35S, Pnos)

barstar : region coding for barstar (as example of coregulating DNA)

Pmin : minimal plant promoter

SEQUENCE LISTING

SEQ ID NO 1:	pTS174
SEQ ID NO 2:	pTS88 HindIII-EcoRI
SEQ ID NO 3:	pVE136 - EcoRI-HindIII
SEQ ID NO 4:	T-DNA of pTCO113

Examples

Example 1 : Coregulating genes in rice

Compact embryogenic callus from rice cultivar Kochihibiki was obtained and transformation of callus cells by electroporation was achieved using the procedures as described in WO 92/096096, particularly in Example 9, except that the transforming DNA consisted of either plasmid pTS174 or plasmids pTS174 and pTS88 in equimolar amounts or more preferably in a 1:3 molar ratio. Prior to transformation, pTS174 and pTS88 were preferably linearized by digestion with

appropriate restriction enzymes. All tissue culture steps were also carried out as decribed in WO 92/09696, example 9.

pTS174 is a pUC19 derived plasmid containing, in its polylinker, the barnase DNA under control of the PE1 promoter (PE1-barnase-3'nos) and the bar gene under control of a 35S promoter (P35S-bar-3'g7). The sequence of pTS174 is given in SEQ ID No 1. pTS88 is a pGEM2 derived plasmid containing, between the HindIII and EcoRI sites of its polylinker, the barstar DNA under control of a 35S promoter (P35S-barstar-3'g7). The sequence of the HindIII-EcoRI fragment of pTS88 is given in SEQ ID No 2. All chimeric genes comprising barstar, barnase, or bar also contained suitable 3' untranslated regions (e.g. of the nopaline synthase gene (3'nos) and gene 7 (3'g7) of Agrobacterium T-DNA).

Plasmids containing a fertility restorer gene comprising the barstar DNA under control of stamen-specific promoter of rice (PE1-barstar), and a herbicide resistance gene comprising the bar gene under control of the 35S promoter (P35S-bar) chimeric genes were used as control.

The results of the transformation experiments is presented in Table 1. In transformation experiments with pTS174 only one normal male-sterile line could be recovered from 48 electroporation cuvettes. In transformation experiments with PTS174 + pTS88, 7 normal male sterile lines could be recovered from 40 cuvettes. Each cuvette contained about 50 callus pieces (approximately 1-2 mm in diameter) of tissue fragments.

In this regard a normal male-sterile plant is understood to be a male-sterile rice plant (i.e. with small, white anthers that do not contain pollen) that is otherwise completely normal (e.g. is female-fertile) and that transmits the male-sterility phenotype to its progeny in accordance with normal Mendelian segregation of the chimeric barnase gene.

From table 1 it is also clear that the advantage of using pTS174 + pTS88 over using pTS174 alone resides in the number of normal regenerated shoots that can be recovered on selective regeneration medium thus attesting to the fact that the P35S-barstar gene affects mainly cells in tissue culture. In this regard it is also important to note that plants containing both the P35S-barstar-3'g7 and the PE1-barnase-3'nos

chimeric genes are male-sterile attesting to the fact that the P35S promoter is not active (or less active than the PE1 promoter) in specific stamen cells (particularly tapetum cells) of rice plants.

Example 2 : Coregulating genes in corn.

Maize plants of lines H99, Pa91 and (Pa91xH99)xH99 ((PxH)xH) were grown in the greenhouse. Type I callus was initiated from immature zygotic embryos of 1 to 1.5 mm in size, which were excised from ears 10 to 14 days after pollination and then plated on MahIVII callus initiation medium (D'Halluin et al, 1992, The Plant Cell 4:1495-1505). Embryogenic callus was removed from the scutella of the embryos and subcultured every 2 to 3 weeks on Mah1VII substrate. Pieces of embryogenic tissue (about 1 to 1.5 mm in diameter) were isolated from actively growing embryogenic callus cultures and were placed on a plate with MahIVII substrate supplemented with 0.2 M mannitol and 0.2 M sorbitol for osmotic pretreatment for 4 hours before bombardment (Vain et al, 1993, Plant Cell Reports 12:84-88). A total amount of about 250 mg of tissue per plate was used in the bombardment experiments. DNA was bombarded into the tissue using the PDS-1000/He Biolistics® device (Bio-Rad). Microcarrier preparation and coating of DNA onto the microcarriers was essentially as described by Sanford et al (1993, In Wu, R. (Ed.). Meth. Enzymol. 217:483-509). The particle bombardment parameters were: target distance: 6 to 9 cm; bombardment pressures: 1100 to 1500 psi; gap distance: 1/4 inches; macrocarrier flight distance: 11 mm. DNA was either linear or circular. The bombarded tissue was removed from the high osmotic medium (between 0 to 24 hours after bombardment) and transferred to selective maintenance medium without caseïne hydrolysate and proline, but containing 10 to 20 mg/l BASTA. The embryogenic callus was subcultured every 2 to 3 weeks for a total period of 6 to 8 weeks and was then transferred to MS medium (Murashige and Skoog, 1962, Physiol. Plant. 15:473-497) containing 3% sucrose, 10-20 mg/l BASTA, and 5 mg/l BAP (for lines H99) or 5 mg/l zeatine (for lines Pa91 or (PxH)xH). The embryogenic tissue was subcultured twice on substrate containing the appropriate cytokinin. Small regenerating plants were

recovered and transferred to MS medium without hormones, but containing 6% sucrose and 10-20 mg/l BASTA. Further developing shoots were transferred to half-strength MS medium with 1.5 % sucrose for further elongation. The resulting plantlets were then transferred to soil in the greenhouse. It was found that after the transformation step, the concentration of BASTA in the culture medium could be reduced down to 2 mg/l.

The following DNA was used. In one set of experiments callus was transformed with plasmid pVE136 which is a pUC19 derived plasmid containing, between the EcoRI and HindIII sites of its polylinker, the barnase DNA under control of the PCA55 promoter (PCA55-barnase-3'nos) and a chimeric P35S-bar-3'nos gene. The sequence of the EcoRI-HindIII fragment of pVE136 is given in SEQ ID. No. 3. In other experiments callus was bombarded with an equimolar mixture of pVE136 and pTS88. pTS88 is the plasmid described in Example 1. In control experiments callus was bombarded with plasmid pDE110 which is a plasmid containing only the P35S-bar-3'nos chimeric gene and is described in WO 92/29696.

The results of the transformation experiments are presented in Table 2. In transformation experiments with pVE136 + pTS88 the number of PAT positive plants, relative to the starting material, is almost twice that obtained in experiments using pVE136 alone.

It is important to note that corn plants containing both the P35S-barstar-3'g7 and the PCA55-barnase-3'nos chimeric genes are male-sterile attesting to the fact that th P35S promoter is not active (or less active than the PCA55 promoter) in stamen cells of corn plants.

Example 3 : Coregulating genes in oilseed rape

Oilseed rape plants (Brassica napus - both spring and winter varieties) were transformed with plasmid pTCO113 using the Agrobacterium mediated transformation procedure essentially as decribed by De Block et al, 1989, Plant Physiol. 91:694-701.

Plasmid pTCO113 is a intermediate cloning vector (T-DNA vector) containing between Agrobacterium T-DNA borders the following genes:

- the bar gene under control of the PSSU promoter
- the barnase gene under control of the PTA29 promoter
- the barstar gene under control of the Pnos promoter.

The sequence of the T-DNA of pTCO113 is presented in SEQ ID. No 4.

Transformation efficiency with pTCO113 was observed to be equal to that obtained with pTHW107 which is a T-DNA vector that is identical to pTCO113 but lacks the Pnos-barstar gene (the nucleotide sequence of pTHW107 is identical to that of SEQ ID No. 4 except that it lacks the nucleotide region 4917-5834).

Oilseed rape plants transformed with pTC0113 were observed to be male-sterile. More precisely, of 31 spring oilseed rape plants regenerated after transformation with pTCO113, 27 plants (87%) were shown to be male-sterile. Of 22 spring oilseed rape plants regenerated after transformation with pTHW107, 20 plants (91%) were shown to be male-sterile (Table 3).

Seeds harvested from male-sterile TO plants pollinated by untransformed male-fertile plants, were grown into T1 plants in the greenhouse. 50% of the plants of each T1 line are expected to carry the male-sterility gene. Plants were analyzed at the time that 50%, respectively 100% of the plants had started flowering. It was observed that plants transformed with pTCO113 have a smaller delay of flowering as compared to plants transformed with pTHW107. This was measured by the ratio of male-fertile (F)/male-sterile (S) plants at the moment that 50% of the plants had started to flower

(Table 3). When all plants flowered, the ratio F/S was 54/46 for both pTCO113 and pTHW107 plants.

The seeds harvested from male-sterile T1 plants of different lines, pollinated by untransformed male-fertile plants, were sown in the field and analyzed with respect to the segregation of the male-sterility genes in the T2 progeny plants. Only 2 out of 7 tested pTHW107 lines, but no less than 12 out of 14 tested pTCO113 lines, showed a normal 1:1 Mendelian segregation (X^2=6.86, $p<0.01$) (Table 3).
It can therefore be conducted that in transformation experiments with pTCO113 a higher percentage of good male-sterile plants was obtained.

TABLE 1

DNA	Total Nr of cuvettes	Regenerants			
	(Nr of experiments)	PAT+[1] normal shoots	PCR+[2]	male-sterility phenotype [3]	progeny analysis[4]
pTS174	48 (9)	1	1/1	1/1	1/1
pTS174 + pTS88	40 (8)	33	24/33	9/24	7/9
FR constructs	23(9)	23	-	-	-

1) Total number of shoots regenerated on PPT (i.e. selective) medium that appeared phenotypically normal
2) Number of PCR+ plants/Number of analyzed PAT+ plants. PCR+ for barnase or barnase/barstar.
3) Number of male-sterile but otherwise normal plants/number of analyzed barnase PCR+ plants
4) Number of phenotypically normal male-sterile plants with good segregation of male-sterile phenotype in progeny/Nr of analyzed male-sterile plants

TABLE 2

DNA	Total Nr of bombarded plates	Regenerants			Progeny analysis
		PAT+[1)]	PCR+[2)] barnase	male-sterility phenotype [3)]	
pVE136	118	68[5)]	34/62	27/34	7/16
pVE136+pTS88	131	141	82/125	64/82	17/34
pDE110[6)]	65	125	-	-	

1) Total number of PAT+ regenerants recovered from all transformation experiments.
2) Number of PCR+ plants/Number of analyzed PAT+ plants
3) Number of male-sterile plants/number of analyzed barnasePCR+ plants
4) Number of male-sterile plants with good segregation (1:1) of male-sterile phenotype in progeny/Nr of analyzed male-sterile plants
5) Number of selected calli was significantly less when compared to calli of transformation experiments containing P35S-barstar
6) Cotransformation experiments using pDE110 in combination with plasmids not comprising cytotoxic genes.

TABLE 3

	T0[1]	T1[2]		T2
		F/S at 50% Flowering	F/S at 100% Flowering	Progeny Analysis[3]
pTHW107	20/22	72% / 28%	54% / 46%	2 / 7
pTCO113	27/31	62% / 38%	54% / 46%	12 / 14

1) T0 : Number of transformed plants that were male-sterile/number of Basta-tolerant plants regenerated after transformation.

2) T1 : percentage of T1 plants with male-fertile flowers (F) / percentage of T1 plants with male-sterile flowers (S) at a time that 50%, respectively 100%, of the T1 plants started to flower. Data from different lines (18 pTHW107 and 17 pTCO113 lines respectively) were pooled.

3) T2: Number of T1 lines that have a normal 1:1 segregation of the male-sterility gene / total numbers of T1 lines that were examined in the field.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: PLANT GENETIC SYSTEMS N.V.
(B) STREET: Plateaustraat 22
(C) CITY: Ghent
(E) COUNTRY: Belgium
(F) POSTAL CODE (ZIP): 9000
(G) TELEPHONE: 32 9 235 84 58
(H) TELEFAX: 32 9 224 06 94
(I) TELEX: 11.361 Pgsgen

(ii) TITLE OF INVENTION: Method to obtain male sterile plants

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6548 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: plasmid pTS174

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..2003
(D) OTHER INFORMATION:/label= vector
/note= "pUC19 derived vector sequences"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (2019..2283)
(D) OTHER INFORMATION:/label= 3'nos
/note= "region containing polyadenylation signal of nopaline
synthase gene of Agrobacterium T-DNA"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (2284..2624)
(D) OTHER INFORMATION:/label= barnase
/note= "region coding for barnase of Bacillus amyloliquefaciens"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (2625..4313)
(D) OTHER INFORMATION:/label= PE1
/note= "promoter of the stamen-specific E1 gene of rice"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:4336..5710
(D) OTHER INFORMATION:/label= P35S
/note= "35S promoter of Cauliflower Mosaic Virus"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:5711..6262
(D) OTHER INFORMATION:/label= bar
/note= "region coding for phosphinothricin acetyl transferase"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:6263..6496
(D) OTHER INFORMATION:/label= 3'g7
/note= "region containing polyadenylation signal of gene 7 of
Agrobacterium T-DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AATTCAAGCT TGACGTCAGG TGGCACTTTT CGGGGAAATG TGCGCGGAAC CCCTATTTGT 60

TTATTTTTCT AAATACATTC AAATATGTAT CCGCTCATGA GACAATAACC CTGATAAATG 120

CTTCAATAAT ATTGAAAAAG GAAGAGTATG AGTATTCAAC ATTTCCGTGT CGCCCTTATT 180

CCCTTTTTTG CGGCATTTTG CCTTCCTGTT TTTGCTCACC CAGAAACGCT GGTGAAAGTA 240

AAAGATGCTG AAGATCAGTT GGGTGCACGA GTGGGTTACA TCGAACTGGA TCTCAACAGC 300

GGTAAGATCC TTGAGAGTTT TCGCCCCGAA GAACGTTTTC CAATGATGAG CACTTTTAAA 360

GTTCTGCTAT GTGGCGCGGT ATTATCCCGT ATTGACGCCG GGCAAGAGCA ACTCGGTCGC 420

CGCATACACT ATTCTCAGAA TGACTTGGTT GAGTACTCAC CAGTCACAGA AAAGCATCTT 480

ACGGATGGCA TGACAGTAAG AGAATTATGC AGTGCTGCCA TAACCATGAG TGATAACACT 540

GCGGCCAACT TACTTCTGAC AACGATCGGA GGACCGAAGG AGCTAACCGC TTTTTTGCAC 600

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GATAAAGTTG CAGGACCACT TCTGCGCTCG GCCCTTCCGG CTGGCTGGTT TATTGCTGAT 840

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CTGGCCTTTT GCTGGCCTTT TGCTCACATG TTCTTTCCTG CGTTATCCCC TGATTCTGTG 1860

GATAACCGTA TTACCGCCTT TGAGTGAGCT GATACCGCTC GCCGCAGCCG AACGACCGAG 1920

CGCAGCGAGT CAGTGAGCGA GGAAGCGGAA GAGCGCCCAA TACGCAAACC GCCTCTCCCC 1980

GCGCGTTGGC CTGATCAGAA TTCATATGCA CGTGTTCCCG ATCTAGTAAC ATAGATGACA 2040

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ACATGCCTTC AGAGAAATGG CATTGACCGT CCATTACAAA CTAACGTACA CTGTATCTGT 3900

TCTTTACGAC ATTGCATGTG GAAAGGATCT GAAGAGATTT CTCCTGGTAC ATAATAATCT 3960

ACTCCTTTGC TACGTTAATA AGAGATGTAA AAACATGCAA CAGTTCCAGT GCCAACATTG 4020

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GGACGATTTC CTCTATCTTT ACGATCTAGG AAGGAAGTTC GAAGGTGAAG GTGACGACAC 4800

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CATTTCATTT GGAGAGGACA CGCTGAAATC ACCAGTCTCT CTCTATAAAT CTATCTCTCT 5700

CTCTATAACC ATGGACCCAG AACGACGCCC GGCCGACATC CGCCGTGCCA CCGAGGCGGA 5760

CATGCCGGCG GTCTGCACCA TCGTCAACCA CTACATCGAG ACAAGCACGG TCAACTTCCG 5820

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TCCCTGGCTC GTCGCCGAGG TGGACGGCGA GGTCGCCGGC ATCGCCTACG CGGGCCCCTG 5940

GAAGGCACGC AACGCCTACG ACTGGACGGC CGAGTCGACC GTGTACGTCT CCCCCCGCCA 6000

CCAGCGGACG GGACTGGGCT CCACGCTCTA CACCCACCTG CTGAAGTCCC TGGAGGCACA 6060

GGGCTTCAAG AGCGTGGTCG CTGTCATCGG GCTGCCCAAC GACCCGAGCG TGCGCATGCA 6120

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TTATGTTTTT GCTTGGACTA TAATACCTGA CTTGTTATTT TATCAATAAA TATTTAAACT 6480

ATATTTCTTT CAAGATGGGA ATTAACATCT ACAAATTGCC TTTTCTTATC GACCATGTAC 6540

GTATCGCG 6548

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1303 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HindIII-EcoRI fragment of pTS88

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..35
(D) OTHER INFORMATION:/label= pGEM2
/note= "polylinker of pGEM2"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:36..694
(D) OTHER INFORMATION:/label= P35S
/note= "35S promoter of Cauliflower Mosaic Virus strain CM1841"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:695..967
(D) OTHER INFORMATION:/label= barstar
/note= "region coding for barstar of Bacillus amyloliquefaciens"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:968..1287
(D) OTHER INFORMATION:/label= 3'g7
/note= "region containing polyadenylation signal of gene 7 of
Agrobacterium T-DNA"

(ix) FEATURE:
(A) NAME/KEY: -

(B) LOCATION:1288..1303
(D) OTHER INFORMATION:/label= pGEM2
/note= "polylinker of pGEM2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AAGCTTGGGC TGCAGGTCGA CTCTAGAGGA TCCCCACTAT TCCAGTATGG ACGATTCAAG 60

GCTTGCTTCA TAAACCAAGG CAAGTAATAG AGATTGGAGT CTCTAAGAAA GTAGTTCCTA 120

CTGAATCAAA GGCCATGGAG TCAAAAATTC AGATCGAGGA TCTAACAGAA CTCGCCGTGA 180

AGACTGGCGA ACAGTTCATA CAGAGTCTTT TACGACTCAA TGACAAGAAG AAAATCTTCG 240

TCAACATGGT GGAGCACGAC ACTCTCGTCT ACTCCAAGAA TATCAAAGAT ACAGTCTCAG 300

AAGACCAAAG GGCTATTGAG ACTTTTCAAC AAAGGGTAAT ATCGGGAAAC CTCCTCGGAT 360

TCCATTGCCC AGCTATCTGT CACTTCATCA AAAGGACAGT AGAAAAGGAA GGTGGCACCT 420

ACAAATGCCA TCATTGCGAT AAAGGAAAGG CTATCGTTCA AGATGCCTCT GCCGACAGTG 480

GTCCCAAAGA TGGACCCCCA CCCACGAGGA GCATCGTGGA AAAAGAAGAC GTTCCAACCA 540

CGTCTTCAAA GCAAGTGGAT TGATGTGATA TCTCCACTGA CGTAAGGGAT GACGCACAAT 600

CCCACTATCC TTCGCAAGAC CCTTCCTCTA TATAAGGAAG TTCATTTCAT TTGGAGAGGA 660

CACGCTGAAA TCACCAGTCT CTCTCTACAA ATCGATGAAA AAAGCAGTCA TTAACGGGGA 720

ACAAATCAGA AGTATCAGCG ACCTCCACCA GACATTGAAA AAGGAGCTTG CCCTTCCGGA 780

ATACTACGGT GAAAACCTGG ACGCTTTATG GGATTGTCTG ACCGGATGGG TGGAGTACCC 840

GCTCGTTTTG GAATGGAGGC AGTTTGAACA AAGCAAGCAG CTGACTGAAA ATGGCGCCGA 900

GAGTGTGCTT CAGGTTTTCC GTGAAGCGAA AGCGGAAGGC TGCGACATCA CCATCATACT 960

TTCTTAATAC GATCAATGGG AGATGAACAA TATGGAAACA CAAACCCGCA AGCTTGGTCT 1020

AGAGGATCCC CCGATGAGCT AAGCTAGCTA TATCATCAAT TTATGTATTA CACATAATAT 1080

CGCACTCAGT CTTTCATCTA CGGCAATGTA CCAGCTGATA TAATCAGTTA TTGAAATATT 1140

TCTGAATTTA AACTTGCATC AATAAATTTA TGTTTTTGCT TGGACTATAA TACCTGACTT 1200

GTTATTTTAT CAATAAATAT TTAAACTATA TTTCTTTCAA GATGGGAATT AACATCTACA 1260

AATTGCCTTT TCTTATCGAC CATGTACGGG CGAGCTCGAA TTC 1303

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3658 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: EcoRI-HindIII fragment of pVE136

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..26
(D) OTHER INFORMATION:/label= pUC19
/note= "polylinker of pUC19"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (28..403)
(D) OTHER INFORMATION:/label= 3'nos
/note= "region containing polyadenylation signal of nopaline
synthase gene of Agrobacterium T-DNA"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (404..739)
(D) OTHER INFORMATION:/label= barnase
/note= "region coding for barnase of Bacillus amyloliquefaciens"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (740..1918)
(D) OTHER INFORMATION:/label= PCA55
/note= "promoter of CA55 gene of Zea mays"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1956..2788
(D) OTHER INFORMATION:/label= P35S
/note= "35S promoter of Cauliflower Mosaic Virus"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:2789..3340
(D) OTHER INFORMATION:/label= bar
/note= "region coding for phosphinothricin acetyl transferase"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:3341..3623
(D) OTHER INFORMATION:/label= 3'nos
/note= "region containing polyadenylation signal of nopaline
synthase gene of Agrobacterium T-DNA"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:3624..3658
(D) OTHER INFORMATION:/label= pUC19
/note= "polylinker of pUC19"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GAATTCGAGC TCGGTACCCG GGGATCTTCC CGATCTAGTA ACATAGATGA CACCGCGCGC 60

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TGCGGGACTC TAATCATAAA AACCCATCTC ATAAATAACG TCATGCATTA CATGTTAATT 180

ATTACATGCT TAACGTAATT CAACAGAAAT TATATGATAA TCATCGCAAG ACCGGCAACA 240

GGATTCAATC TTAAGAAACT TTATTGCCAA ATGTTTGAAC GATCTGCTTC GGATCCTCTA 300

GAGCCGGAAA GTGAAATTGA CCGATCAGAG TTTGAAGAAA AATTTATTAC ACACTTTATG 360

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: T-DNA of plasmid pTCO113

(ix) FEATURE:
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(B) LOCATION:complement (1..25)
(D) OTHER INFORMATION:/label= RB
/note= "right border of Agrobacterium T-DNA"

(ix) FEATURE:
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(B) LOCATION:complement (98..330)
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(ix) FEATURE:
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(B) LOCATION:complement (883..2608)
(D) OTHER INFORMATION:/label= Pssu
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(ix) FEATURE:
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(B) LOCATION:complement (3368..4877)
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(ix) FEATURE:
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(ix) FEATURE:
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(D) OTHER INFORMATION:/label= barstar
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(ix) FEATURE:
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(D) OTHER INFORMATION:/label= LB
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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CLAIMS

1. A plant having in the nuclear genome of its cells foreign DNA comprising

- a male-sterility gene comprising:
  - a male-sterility DNA encoding a sterility RNA, protein or polypeptide which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell, and,
  - a sterility promoter directing expression of the male-sterility DNA selectively in specific stamen cells, especially in anther cells, particularly in tapetum cells, of said plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter; and
- a coregulating gene comprising:
  - a coregulating DNA encoding a coregulating RNA, protein or polypeptide which is capable, when produced in plant cells wherein said sterility RNA, protein or polypeptide is produced, of sufficiently preventing the activity of said sterility RNA, protein or polypeptide, and preferably
  - a promoter directing expression of said coregulating DNA in non-stamen cells, preferably at least in the majority of non-stamen cells, while directing low-level expression, preferably not directing expression, in said specific stamen cells, or
  - a promoter consisting of a minimal promoter element, preferably of a promoter normally expressed in plant cells, particularly whereby said coregulating DNA is under control of enhancer elements in the nuclear genome of said plant.

whereby said coregulating DNA is in a transcriptional unit which is different from the transcriptional unit of said sterility DNA.

2. A plant according to claim 1, wherein said male-sterility gene and said coregulating gene are adjacent to one another.

3. The plant of claim 1 in which said sterility DNA encodes barnase or a variant thereof and said coregulating DNA encodes barstar. or a variant thereof.

4. The plant of any one of claims 1 to 3 in which said sterility promoter is PTA29 and said coregulating promoter is Pnos.

5. The plant of any one of claims 1 to 4 in which said plant is a dicot plant, particularly a Brassica plant.

6. The plant of any one of claims 1 to 3 in which said sterility promoter is PCA55, PE1, PT72 or PT42, and said coregulating promoter is P35S.

7. The plant of any one of claims 1 to 3 or 6 in which said plant is a monocot plant, particularly corn or rice.

8. The plant of any one of claims 1 to 3 in which said coregulating promoter is a minimal promoter operable in plant cells.

9. A cell of a plant according to any one of claims 1 to 8.

10. A process to obtain male-sterile plant which comprises :

- transforming the nuclear genome of plant cells with a foreign DNA comprising a male-sterility gene comprising:
  - a male-sterility DNA encoding a sterility RNA, protein or polypeptide which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell, and,
  - a sterility promoter capable of directing expression of the male-sterility DNA selectively in specific stamen cells, especially in anther cells, particularly in tapetum cells, of said plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter, and
- regenerating plants transformed with said foreign DNA from said transformed cells, which method is characterized by including in said foreign DNA a coregulating RNA,

protein or polypeptide which is capable, when produced in plant cells wherein said sterility RNA, protein or polypeptide is produced, of sufficiently preventing the activity of said sterility RNA, protein or polypeptide, said coregulating DNA preferably being under the control of a promoter including:

- a promoter capable of directing expression of said coregulating DNA in non-stamen cells, preferably at least in the majority of non-stamen cells, while directing low-level expression, preferably not directing expression, in said specific stamen cells, or
- a promoter consisting of a minimal promoter element, preferably of a promoter normally expressed in plant cells, particularly whereby said coregulating DNA is capable of being placed under control of enhancer elements in the nuclear genome of said plant after integration of said foreign DNA in said plant genome,

whereby said coregulating DNA is in a transcriptional unit which is different from the transcriptional unit of said sterility DNA, and provided that, when said coregulating DNA is not under control of a promoter capable of directing expression in plant cells, said coregulating gene is located in said foreign DNA in such a way that after insertion in the plant genome, the coregulating DNA is capable of being placed under the control of plant promoter sequences present in the DNA surrounding said foreign DNA in said plant genome.

11. The process of claim 10 in which said sterility DNA encodes barnase or a variant thereof and said coregulating DNA encodes barstar or a variant thereof.

12. The process of claim 10 or 11 in which said sterility promoter is PTA29 and said coregulating promoter is Pnos.

13. The process of any one of claims 10 to 12 in which said male-sterile plant is a dicot plant, particularly a Brassica plant.

14. The process of claims 10 or 11 in which said sterility promoter is PCA55, PE1, PT72 or PT42, and said coregulating promoter is P35S.

15. The process of claims 10, 11 or 14 in which said male-sterile plant is a monocot plant, particularly corn or rice.

16. The process of claim 10 or 11 in which said coregulating promoter is a minimal promoter operable in plant cells.

17. A plant obtained by the process according to any one of claims 10 to 16.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC1/EP 96/00722

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N5/10 A01H1/02 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,91 09957 (DU PONT) 11 July 1991 see example 12 ---	1-17
A	WO,A,93 19188 (MAX PLANCK GESELLSCHAFT) 30 September 1993 cited in the application see page 13, line 8 - page 14, line 27; examples 3,4 ---	1-17
A	WO,A,92 21757 (PLANT GENETIC SYSTEMS NV) 10 December 1992 cited in the application see claim 6 ---	1-17
A	WO,A,93 10251 (MOGEN INT) 27 May 1993 see the whole document --- -/--	1-17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 May 1996	07.06.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Maddox, A

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC./EP 96/00722

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 08291 (PLANT GENETIC SYSTEMS) 29 April 1993 see page 10, line 9 - line 25 see page 14, line 7 - line 24 -----	1-17

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter onal Application No
PC./EP 96/00722

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		AU-B-	6974791	24-07-91
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		HU-A-	70264	28-09-95
		JP-T-	7500970	02-02-95
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